ECOTOXICOLOGY AND ENVIRONMENTAL SAFETY 2, 249–255 (1978)

The Use of *Artemia* Nauplii for Toxicity Tests—A Critical Analysis

PATRICK SORGELOOS,[1] CLAIRE REMICHE-VAN DER WIELEN, AND GUIDO PERSOONE

Laboratory for Biological Research in Aquatic Pollution, Artemia Reference Center, State University of Ghent, J. Plateaustraat 22, 9000 Ghent, Belgium, and Ministry of Public Health, Institute for Hygiene and Epidemiology, J. Wytsmanstraat 14, 1050 Brussels, Belgium

Received August 1, 1978

Because of their easy hatching from dry cysts and their year-round availability, nauplii of the brine shrimp *Artemia salina* are most convenient test organisms for toxicity studies. The bioassays mentioned in literature have, however, mostly been carried out with larvae of which neither the exact instar stage nor the geographical origin is known. The incubation of the cysts should always be carried out under strictly controlled temperature conditions since both the onset of hatching and the length of the hatching period are temperature-dependent. Moreover, the experiments should only be carried out with populations of nauplii of the same stage of development since it is demonstrated that second and third instars are significantly more sensitive to toxicants than first instar larvae. It is demonstrated that the sensitivity to chemicals varies from one geographical *Artemia* strain to another.

If one considers the year-round and worldwide availability of dry cysts of the brine shrimp *Artemia salina*, it is quite surprising that this organism has not been utilized more extensively for toxicity studies. *Artemia* cysts can indeed be stored for years without losing their viability, and upon immersion in seawater, nauplii will hatch within a period of 1 to 2 days. As such, *Artemia* has a unique advantage over all other zooplankters as a bioassay organism since it does not require any maintenance or stock culturing.

In the first larval stage (named stage I_3 by D'Agostino, 1965, and instar I by Hentschel, 1968) the digestive tract of the nauplius is not in contact yet with the external medium (Benesch, 1969), and the larva only consumes its yolk. After a certain period of time (which as shown by Hentschel, 1968, depends on the incubation temperature), the nauplius molts into the second larval stage (stage I_4 of D'Agostino or instar II of Hentschel) and starts feeding on particulate matter (Reeve, 1963). The molting into the further larval stages is dependent on both temperature and quality and quantity of food. When not fed, *Artemia* larvae will die during the third or fourth instar stage.

In most of the literature references dealing with *Artemia* bioassays (Michael *et al.*, 1956; Corner and Sparrow, 1956, 1957; Hood, 1960; Wiseley and Blick, 1967; Tarzwell, 1969; Brown and Ahsanullah, 1971; Harwich and Scott, 1971; Navy report, 1973; Zilloux *et al.*, 1973; Price *et al.*, 1974) relatively little emphasis was put on

[1] "Aangesteld Navorser" at the National Foundation for Scientific Research (NFWO).

0147-6513/78/0234-0249$02.00/0

hatching conditions. As will be shown hereafter the experimental data obtained are liable to fluctuation and are hardly reproducible.

To assess the influence of temperature on the early development of brine shrimp and its eventual consequences on the sensitivity of the test organisms, we carried out experiments on the hatching and molting rate at different temperatures and on the toxicity of a chemical compound on the first, second, and third instars.

Considering the differences in biological characteristics of *Artemia* cysts of different strains (Sorgeloos *et al.*, 1976; Claus *et al.*, 1977) we also compared the sensitivity of the first instar nauplii of *Artemia* races from four different geographical sites.

HATCHING AND MOLTING RATE AT 20 AND 24°C

One gram of *Artemia* cysts, from San Francisco Bay (California, U.S.A.) was hydrated in 500 ml of artificial seawater (formula of Dietrich and Kalle, 1963) in cylindroconical glass tubes and incubated in warm-water baths at either 20 or 24°C.

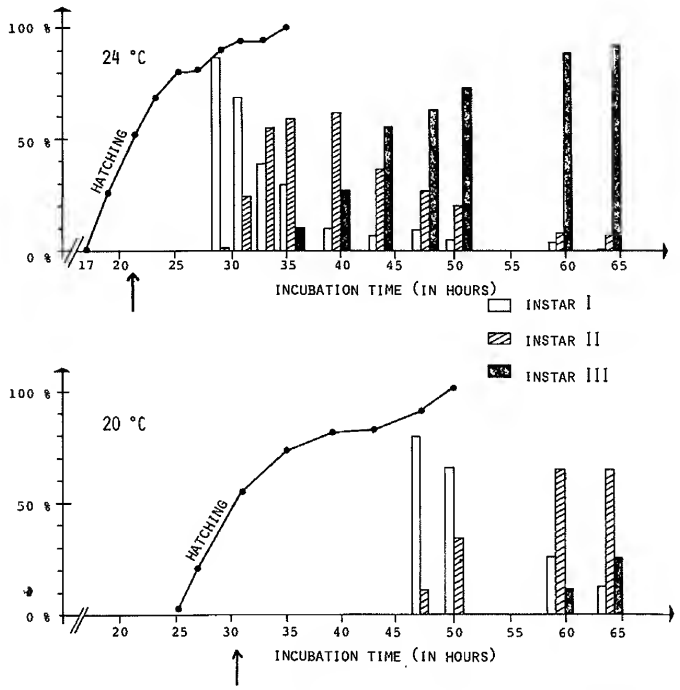

FIG. 1. Influence of the water temperature (20 and 24°C) and of the incubation period on the hatching and the percentage of first, second and third instar larvae.

Since light has a triggering effect on the onset of the hatching (Sorgeloos, 1973) the tubes were illuminated by a fluorescent light tube for 1 hr. Air was bubbled through the suspension by a glass tube extending to the bottom of the hatching vessel to keep all the cysts in continuous motion (Sorgeloos and Persoone, 1975). The experiment was set up in duplicate.

At regular time intervals small samples were taken to determine the percentage hatching and the exact instar stage of the larvae. From Fig. 1 which shows the evaluation of the hatching and of the proportions of the different larval stages, it is clear that the rate of hatching is much influenced by temperature: 50% of the cysts hatched within 30 hr at 20°C; at 24°C this percentage was already obtained after 21 hr (see arrows).

With regard to the molting rate it appears that at 24°C more than 60% of the larvae had already molted into the second instar stage 16 hr after hatching, whereas at 20°C it took 35 hr to arrive at the same figure.

DIFFERENCES IN SENSITIVITY OF FIRST, SECOND, AND THIRD INSTAR NAUPLII

Artemia cysts from the Great Salt Lake (Utah, U.S.A.) were incubated at 28°C in artificial seawater following the procedure outlined above. To obtain a population consisting only of first instar nauplii the larvae were separated from the unhatched cysts, empty cyst shells and debris within 2 hr after the first free-swimming nauplii were observed. The separation based on the principle of the phototactic migration of the larvae was carried out in a separator box (Persoone and Sorgeloos, 1972). One-third of the population was used immediately for the test on the first instars. The rest was incubated overnight at 20°C. The next morning all the animals had molted into the second instar stage. Half of this population was used to run the test on second instars, and after an incubation of another 10 hr the last part of the experiment could be started on the third instar larvae.

The bioassays were carried out with chromic acid as toxicant. Small glass Petri dishes were used containing 10 ml of artificial seawater with concentrations of 100, 80, 60, 40, 20, 15, 10, 5, and 1 ppm of chromic acid. Each petri dish was inoculated with 10 nauplii. The tests were run in triplicate.

After an incubation of 1 hr at 28°C the number of dead larvae was counted in each Petri dish, and the data for the three parallels were averaged. The 1-hr LD_{50} concentrations for the three instar stages were calculated following the straight-line graphical interpolation method (Standard methods, 1971). From Fig. 2 it clearly appears that the first instar larvae are significantly more resistant to chromic acid than the second and third instar stages which have roughly the same sensitivity (LD_{50} of 30 vs 7.4 and 8.2 ppm, respectively). The obvious difference in the lethal doses between first stage versus second and third stage larvae is probably due to the fact that in freshly hatched nauplii the epithelium of the digestive tract is not in contact with the external medium, whereas it is well exposed as soon as the animals molt into the second instar stage.

Our results corroborate and at the same time explain the findings of Wiseley and Blick (1967) who performed bioassays with *Artemia* larvae of increasing age and wrote "the resistance of newly hatched larvae was high and inconsistent at first, but decreased during the first 20 hours to a low and relatively constant level that was maintained for the next 60 hours." As these authors performed their tests at 22°C, it took from 15 to 18 hr before the first instar larvae molted to the second stage. From their graph, which expresses the time–lethal dose effect for a 9.3×10^{-2} M copper

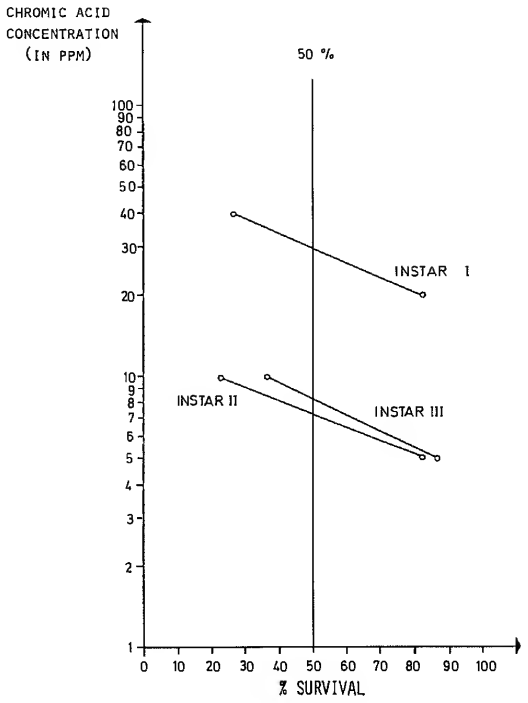

FIG. 2. LD_{50} values for instar I, II, and III *Artemia* larvae from Great Salt Lake (Utah, U.S.A.).

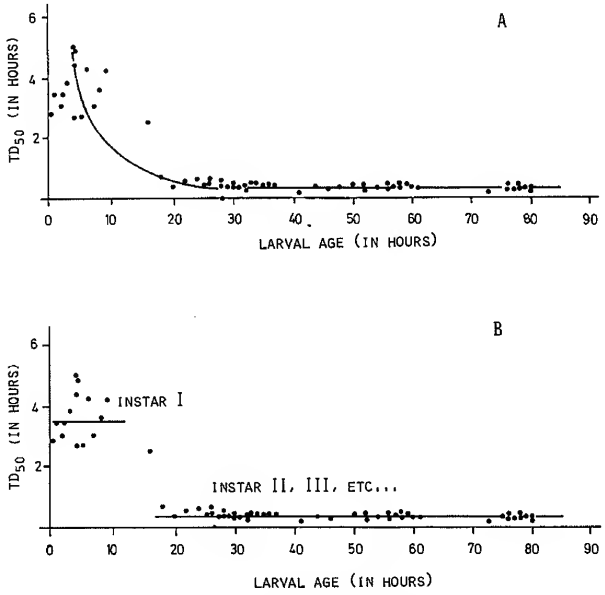

FIG. 3A. TD_{50} values for *Artemia* nauplii of increasing age, exposed to a 9.3×10^{-2} M copper concentration at 22°C (after Wiseley and Blick, 1967). (B) Redrawing of the above graph to accentuate the clearcut difference in sensibility between first and second + third instar larvae.

concentration, it appears that the TD_{50} at 22°C, drops from about 2–5 to 0.2–0.6 hr for an experimentation time of less than 10 hr and more than 20 hr, respectively (Fig. 3A).

In view of our results, the TD_{50} line drawn by Wiseley and Blick is in our opinion somewhat misleading and would be better drawn with a clearcut separation between the two horizontal lines to accentuate the different sensitivity between first instars and older larvae: one line at ±3.5 hr, extending to the 15- to 18-hr larval age and the other one at 0.5 hr starting at ±18 hr (Fig. 3B).

TOXICITY TEST ON FIRST INSTAR NAUPLII FROM FOUR DIFFERENT GEOGRAPHICAL STRAINS

Artemia cysts from San Francisco Bay (California, U.S.A.), Great Salt Lake (Utah, U.S.A.), Burgas-Pomorije (Bulgaria), and the Federal Republic of China (Tsingtao?) were incubated in artificial seawater at 28°C in the same experimental set-up as described previously.

TABLE 1

MORTALITY OF INSTAR I NAUPLII FROM FOUR DIFFERENT GEOGRAPHICAL STRAINS AFTER A 1-HR EXPOSURE TO LUGOL (TWO PARALLELS WITH 10 NAUPLII EACH)

Concentration of lugol (ppm)	San Francisco Bay	Great Salt Lake	Bulgaria	China
500	10	10	10	10
	10	10	10	10
400	10	8	10	9
	10	9	10	9
300	8	4	9	5
	7	4	8	6
200	3	0	2	0
	3	0	3	1
0	0	0	0	0
	0	0	0	0

It has been proven (Sorgeloos, 1975) that there are considerable differences between the four geographical strains in the time needed to complete their embryonic development. To achieve a 50% hatch, it takes 18, 24, 36, and 48 hr for the San Francisco, Utah, Burgas-Pomorije, and China strains, respectively. Ten first instar larvae were transferred to Petri dishes containing 500, 400, 300, 200, and 0 ppm of a Lugol's solution in 10 ml of artificial seawater.

The experiments were carried out in duplicate. From the averaged survival data (Table 1), the calculated LD_{50} values after 1 hr of contact with the toxicant, are as follows: San Francisco Bay, 240 ppm; Great Salt Lake, 320 ppm; Burgas-Pomorije, 240 ppm; and Tsingtao, 290 ppm.

From these results, it is clear that even under the given (constant) experimental temperature–salinity conditions the sensitivity of first instar nauplii differs markedly among different strains.

These results can only strengthen the recommendation of Zilloux *et al.* (1973) to select one specific geographical strain as a standard, especially since it has been shown (Sorgeloos *et al.*, 1976) that the optimal temperature–salinity ranges for survival of the larvae also vary very much from one race to another.

DISCUSSION AND CONCLUSIONS

As already underlined in the introduction there can be no doubt that *Artemia salina* is one of the most, if not *the* most, convenient organism for toxicity tests on microscopical invertebrates. The literature available as well as the results given above demonstrate, however, that a number of precautions must be taken with regard to the reproducibility of the results. Factors which should be kept under strict control are the exact origin of the strain, the temperature during incubation and hatching, the moment of harvesting of the larvae, the period of time between the harvest and the start of the bioassay, and the temperature and salinity of the medium during the test.

Considering the changes in sensitivity of larvae of different morphological development (especially from instar I to II) and also considering the fact that any test carried out with larvae from the third larval stage on will imply feeding the organisms prior to or during the test (which will complicate both the experiment and the interpretation), we recommend that bioassays with *Artemia* larvae be carried out only as short-term toxicity tests (one to a few hours) and only on freshly hatched nauplii. Any deviation from this rule will automatically lead to utilization of populations of mixed morphological stages with different sensitivities.

In view of the endeavors made recently at the international level to standardize toxicity tests, research is now in progress in our laboratory to work out a simple standardized bioassay with first instar *Artemia* larvae for routine purposes.

REFERENCES

BENESCH, R. (1969). Zur Ontogenie und Morphologie von *Artemia salina* L. *Zool. Jahrb. Anat.* **86,** 307–458.

BROWN, B., AND AHSANULLAH, M. (1971). Effect of heavy metals on mortality and growth. *Mar. Pollut. Bull.* **2,** 182–187.

CLAUS, C., BENIJTS, F., AND SORGELOOS, P. (1977). Comparative study of different geographical strains of the brine shrimp *Artemia salina*. In *Fundamental and Applied Research on the Brine Shrimp Artemia salina* (L.) *in Belgium. Special Publication No. 2.* pp. 91–105. (E. Jaspers, ed.). European Mariculture Society. Institute for Marine Scientific Research, Bredene, Belgium.

CORNER, E. D. S., AND SPARROW, B. W. (1956). The modes of action of toxic agents. I. Observations on the poisoning of certain crustaceans by copper and mercury. *J. Mar. Biol. Assoc. UK* **35,** 531–548.

CORNER, E. D. S., AND SPARROW, B. W. (1957). The modes of action of toxic agents. II. Factors influencing the toxicities of mercury compounds to certain crustacea. *J. Mar. Biol. Assoc. UK* **36,** 459–472.

CROGHAN, P. C. (1958). The mechanism of osmotic regulation in *Artemia salina* (L.): The physiology of the gut. *J. Exp. Biol.* **35,** 243–249.

D'AGOSTINO, A. S. (1965). Comparative studies of *Artemia salina* (Development and Physiology). New York University, Ph.D. thesis, University Microfilm 66–5653. University Microfilms, Inc., Ann Arbor, Michigan.

DIETRICH, G., AND KALLE, K. (1963). *General Oceanography.* Interscience, New York.

GILCHRIST, B. M. (1960). Growth and form of the brine shrimp *Artemia salina* (L.) *Proc. Zool. Soc. London* **134,** 221–235.

HARWICH, J., AND SCOTT, P. M. (1971). Brine shrimp (*Artemia salina* L.) larvae as a screening system for fungal toxins. *Appl. Microbiol.* **21,** 1011–1016.

HENTSCHEL, E. (1968). Die postembryonalen Entwicklungsstadien von *Artemia salina* LEACH bei verscheidenen Temperaturen (Anostraca, Crustaceae). *Zool. Anz.* **180,** 372–384.

HOOD, D. W. (1960). Measurement of toxicity of organic wastes to marine organisms. *J. Water Pollut. Cont. Fed.* **32,** 982.

MICHAEL, A. S., THOMPSON, C, G., AND ABRAMOVITZ, M. (1956). *Artemia salina* as a test-organism for bioassay. *Science* **123,** 464.

PERSOONE, G., AND SORGELOOS, P. (1972). An improved separator box for *Artemia* nauplii and other phototactic invertebrates. *Helgol. Wiss. Meeresunters.* **23,** 243–247.

PRICE, K. S., WAGGY, G. T., AND CONWAY, R. A. (1974). Brine shrimp bioassay and seawater BOD of petrochemicals. *J. Water Pollut. Contr. Fed.* **46,** 63–77.

REEVE, M. R. (1963). The filter-feeding of *Artemia*. II. In suspension of various particles. *J. Exp. Biol.* **40,** 207–214.

SORGELOOS, P. (1973). First report on the triggering effect of light on the hatching mechanism of *Artemia salina* dry cysts. *Mar. Biol.* **22,** 75–76.

SORGELOOS, P. (1975). De invloed van abiotische en biotische faktoren op de levenscyclus van het pekelkreeftje *Artemia salina* L. Ph.D. thesis (not published), State University of Ghent.

SORGELOOS, P., BAEZA-MESA, M., BENIJTS, F., AND PERSOONE, G. (1976). Current research on the culturing of the brine shrimp *Artemia salina* L. at the State University of Ghent, Belgium. In *Proceedings 10th European Symposium on Marine Biology,* Ostend (Belgium), September 17–23, 1975. (G. Persoone, and E. Jaspers, eds.), Vol. 1, pp. 473–495. Universa Press, Wetteren, Belgium.

SORGELOOS, P., AND PERSOONE, G. (1975). Technological improvements for the cultivation of invertebrates as food for fishes and crustaceans. II. Hatching and culturing of the brine shrimp *Artemia salina* L. *Aquaculture* **6,** 303–317.

Standard methods for the examination of water and wastewater, 1971. American Public Health Association, American Water Works Association, W.P.C.F., 13th Ed. Washington, D.C.

TARZWELL, C. M. (1969). Standard methods for the determination of relative toxicity of oil dispersants and mixtures of dispersants and various oils to aquatic organisms. In *Proceedings. Joint Conference on Prevention and Control of Oil Spills* (A.P.I.–F.W.P.C.A.), pp. 179–186.

U.S. NAVY (1973). Military specification; Emulsifier, oil-slick. MIL-E-22864 B (NAVY).

WISELEY, B., AND BLICK, R.A.P. (1967). Mortality of marine invertebrate larvae in mercury, copper and zinc solutions. *Aust. J. Mar. Freshwater. Res.* **18,** 63–72.

ZILLIOUX, E. J., FOULK, H. R., PRAGER, J. C., AND CARDIN, J. A. (1973). Using *Artemia* to assay oil dispersant toxicities. *J. Water Pollut. Contr. Fed.* **45,** 2389–2369.